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The role of EF-hand in calmodulin binding of voltage-gated Cav2.1 and Cav2.2 calcium channels

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THE ROLE OF EF-HAND IN CALMODULIN BINDING OF VOLTAGE-GATED CAV2.1 AND CAV2.2 CALCIUM CHANNELS

by

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DEDICATION

"To Him who is able to keep you from falling and
to present you before His glorious presence without fault and with great joy—
to the only God our Savior be glory, majesty, power and authority,
through Jesus Christ our Lord,
before all ages, now and forevermore! Amen."
Jude 1:24-25 (NIV)

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THE ROLE OF EF-HAND IN CALMODULIN BINDING OF VOLTAGE-GATED CAV2.1 AND CAV2.2 CALCIUM CHANNELS

DANIEL HYEONGJIN SOH

ABSTRACT

Voltage-gated $Ca_v2.1$ (P/Q-type) and $Ca_v2.2$ (N-type) channels are two closely related calcium channels that play indispensable roles in signal transduction pathways by regulating neurotransmitter release. Despite having highly conserved amino acid sequences, they are differentially modulated by calmodulin, which mediate two important feedback mechanisms known as Ca^{2+}-dependent inactivation (CDI) and Ca^{2+}-dependent facilitation (CDF). These dual regulatory mechanisms contribute to synaptic plasticity, but only CDI is observed in $Ca_v2.2$ channel, while both CDI and CDF are present in $Ca_v2.1$ channel. Previously, it was hypothesized that the lack of CDF in $Ca_v2.2$ channel is due to the pre-IQ-IQ domain of the channel's lower binding affinity for calmodulin compared to that of $Ca_v2.1$ channel. Now that the EF-hand domain of calcium channels is identified as one of the two minimally required molecular determinants that are responsible for supporting CDF in $Ca_v2.1$ channel and preventing CDF in $Ca_v2.2$ channel, it was necessary to determine the role of EF-hand domain in calmodulin binding of $Ca_v2.1$ and $Ca_v2.2$ channels. Using pull-down binding assays, this study finds that the EF-hand domain enhances calmodulin binding to the proximal C-terminal domain of $Ca_v2.2$ channel, which suggests that the lack of CDF in $Ca_v2.2$ does not result from the channel's weak

interaction with CaM, but from the EF-pre-IQ-IQ domain of the channel's inability to allow calmodulin from fully exerting its effects.

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LIST OF ABBREVIATIONS

Ca^{2+} ..... Calcium (ions)

$[Ca^{2+}]$ ..... Calcium concentration

CaM ..... Calmodulin

CBD ..... Calmodulin Binding Domain

CDF ..... Calcium-Dependent Facilitation

CDI ..... Calcium-Dependent Inactivation

CTD ..... C-Terminal Domain

dCT ..... Distal C-terminal Domain

E. coli ..... *Escherichia coli*

HVA ..... High-Voltage Activated

IPTG ..... Isopropyl β-D-1thiogalactopyranoside

LB ..... Luria Broth

LVA ..... Low-Voltage Activated

pCT ..... Proximal C-terminal Domain

PMSF ..... Phenylmethylsulfonyl Fluoride

rCaM ..... Rat Calmodulin

VGCC ..... Voltage-gated Calcium Channel

INTRODUCTION

Overview of the Voltage-gated Calcium Channels

Calcium ions (Ca^{2+}) are essential signaling molecule that can act as both first and second messengers in various somatosensory signal transduction pathways, controlling vital activities of eukaryotic cells ranging from cell growth to proliferation, differentiation, hormonal regulation, metabolism, contractile and motile function, and apoptosis (Brini et al., 2013). At the center of calcium signaling are the voltage-gated calcium channels (VGCCs), which are a group of ion channels that allow Ca^{2+} influx in a voltage-dependent manner, thereby transducing electrochemical signals to intracellular Ca^{2+} signals (Bergsman et al., 2000). VGCCs are composed of a voltage-sensing, pore-forming Ca_v α_1 core subunit and auxiliary subunits including $\alpha_2\delta$ and β as shown in **Figure 1** (Catterall et al., 2005).

VGCCs are found in the plasma membrane of all excitable cells as well as in a wide variety of non-excitable cells, and they influence a vast amount of physiological and biochemical processes including synaptic transmission, gene transcription, and muscle contraction, by strictly and precisely regulating intracellular calcium concentration ($[Ca^{2+}]$) (Cain et al., 2011; Lee and Catterall, 2005).

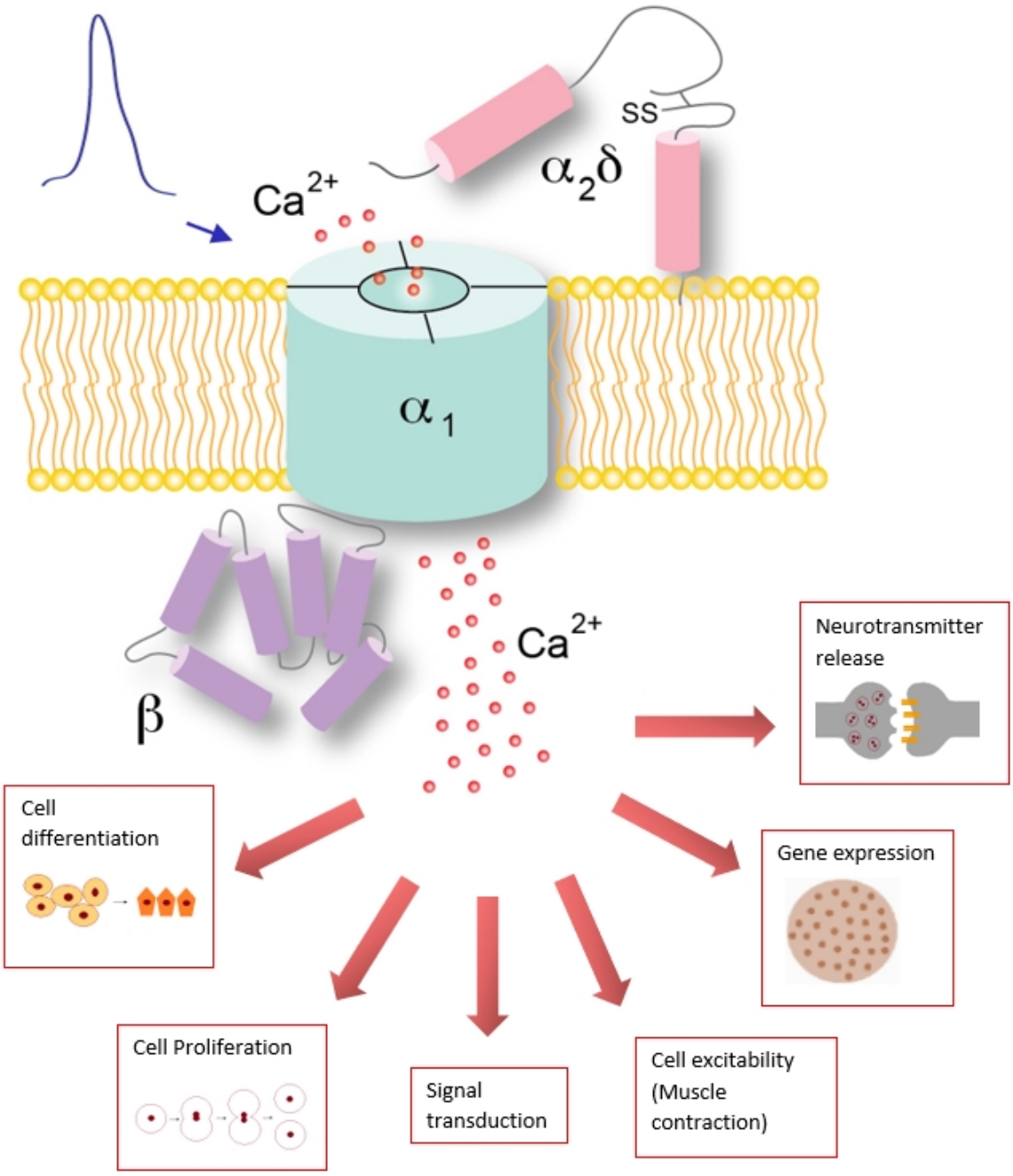
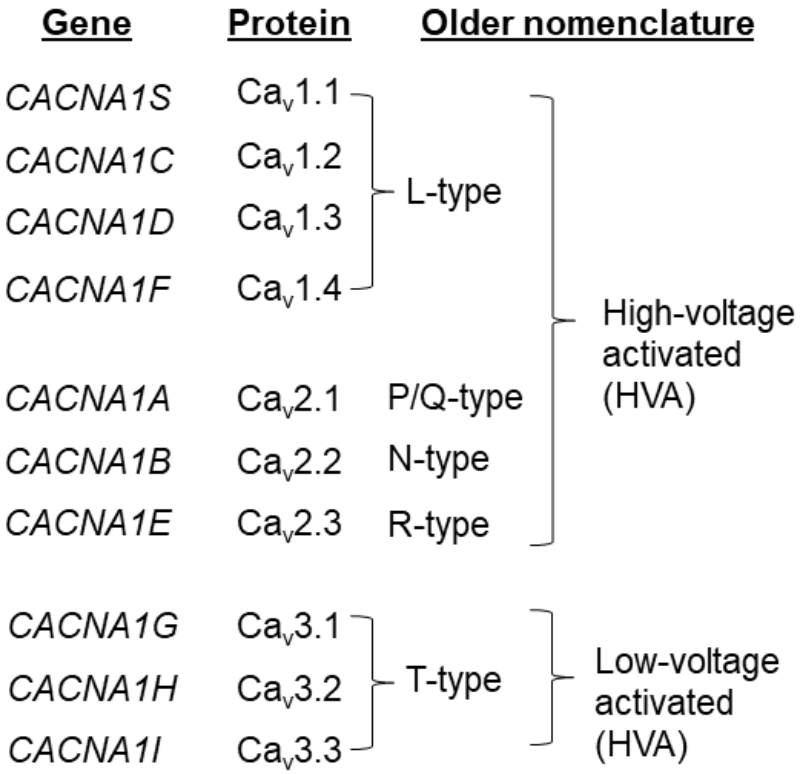


Figure 1. The Structure of Voltage-gated Calcium Channels. Voltage-gated calcium channels are multi-subunit complexes composed of a pore-forming α_1 subunit and two auxiliary $\alpha_2\delta$ and β subunits. VGCCs regulate intracellular [Ca^{2+}] and influences cell excitability and signal transduction.

There are many different types of VGCCs, and they can be broadly categorized into two groups based on the level of activation threshold: high-voltage activated (HVA) channels and low-voltage activated (LVA) channels, which are with and without auxiliary subunits, respectively (Carbone and Lux, 1984). Currently, there are three major types of VGCCs—Ca_v1, Ca_v2, and Ca_v3, each with its own several members, and they can be further classified into five different subtypes—L, N, P/Q, R, and T—depending on biophysical and pharmacological characteristics, many of which are determined primarily by the specific isoform of Ca_v α_1 subunit (Simms and Zamponi 2014).

There are ten distinct human genes that encode Ca_v α_1 subunits (shown in **Table 1**), and there are four genes that encode the intracellular β subunit, which is known to affect gating properties and increase cell surface trafficking (Brice and Dolphin, 1999; Buraei and Yang, 2010). Also, there are four genes that encode the $\alpha_2\delta$ subunit, which is attached to the extracellular portion of the membrane and is known to increase cell surface density of the channel (Cantí et al., 2005).

Table 1: Genes that Encode Different Types of Voltage-gated Calcium Channels. There are ten known genes that encode α_1 subunit of VGCCs. VGCCs can be classified according to different current types, which are based on distinct physiological and pharmacological characteristics.



The alternative splicing of the Ca_v α_1 subunit along with the differential expression of its ancillary subunits contribute to the electrophysiological and functional diversity of the channel (Catterall, 2011). Different type of VGCCs are expressed in different type of cells and tissues, regulating and exhibiting specific subcellular functions. For example, channels that are responsible for excitation-contraction coupling are localized in muscles, while channels that are responsible

for neurotransmitter release are expressed in the presynaptic terminal of neurons. Also, different types of VGCCs are associated with the pathophysiology of different hereditary diseases as summarized in **Table 2** (Catterall, 2005; Yamakage et al., 2002; Snutch, 2009; Striessnig, 2016).

This study focuses on $Ca_v2.1$ (P/Q-type) and $Ca_v2.2$ (N-type) channels, which are closely related, widely expressed in neuronal tissues, and heavily involved in synaptic transmission (Dunlap et al., 1995; Olivera et al., 1994; Catterall 2000). The $Ca_v2.1$ channel is primarily responsible for regulating neurotransmitter release at most synapses of the nervous system, while $Ca_v2.2$ channel, which is primarily responsible for nociception, plays a supporting role to the $Ca_v2.1$ channel at many synapses of the central nervous system (Wheeler et al., 1994; Forsythe et al., 1998; Mintz et al., 1995; Hatakeyama et al., 2001). In humans, $Ca_v2.1$ channel defect is associated with familiar hemiplegic migraine type I and ataxia (Striessnig, 2016), while $Ca_v2.2$ channel defect may be associated with myclonus-dystonia syndrome (Mencacci et al., 2015). In $Ca_v2.1$ knockout mice, ataxia, seizures, and premature death are observed (Jun et al., 1999), while no apparent phenotype changes are observed other than sympathetic nerve dysfunction and high pain threshold in mice with genetic deficiency of $Ca_v2.2$ channel (Ino et al., 2001; Hatakeyama et al., 2001).

Table 2: Major Localizations, Functions, and Diseases Associated with Voltage-gated Calcium Channels (Catterall, 2005; Snutch, 2009; Striessnig, 2016).

Ca_v Channel	Localization	Physiological Functions	Diseases Associated
$Ca_v1.1$	Skeletal muscle; transverse tubules	Excitation-contraction coupling; transcription regulation	Hypokalemic periodic paralysis
$Ca_v1.2$	Cardiac muscle; smooth muscle; endocrine cells; neurons	Excitation-contraction coupling; gene transcription; hormone secretion	Timothy syndrome; Brugada syndrome
$Ca_v1.3$	Cardiac atrial myocytes; endocrine cells; neurons; cochlear hair cells	Hormone and neurotransmitter release; hearing; gene transcription; pacemaking	Sinoatrial node dysfunction; deafness
$Ca_v1.4$	Retinal rod and bipolar cells	Neurotransmitter release in visual system	Stationary night blindness; various X-linked retinal disorders
$Ca_v2.1$	Nerve terminals and dendrites; neuroendocrine cells	Neurotransmitter release; dendritic signaling; hormone release	Familial hemiplegic migrane; cerebral ataxia;
$Ca_v2.2$	Neve terminals and dendrites; neuroendocrine cells	Neurotransmitter release from spinal nociceptive neurons; dendritic signaling	
$Ca_v2.3$	Neuronal cell bodies and dendrites	Repetitive firing; selective neurotransmitter release	
$Ca_v3.1$	Neuronal cell bodies and dendrites; cardiac and smooth muscle	Pacemaking; repetitive firing; cellular differentiation	Spinocerebellar ataxia
$Ca_v3.2$	Neuronal cell bodies and dendrites; cardiac and smooth muscle	Pacemaking; repetitive firing	Absence seizures
$Ca_v3.3$	Neuronal cell bodies and dendrites	Pacemaking; repetitive firing	

Calmodulin Regulation of Voltage-gated Calcium Channels

Since VGCCs have a profound influence in the human body, it is crucial to examine and understand how these channels are regulated by various molecules as well as how they interact with them. Their study is important for the discovery of new ways to treat diseases associated with Ca^{2+} channel defect. There are many direct and in-direct modes in which VGCCs are regulated, and there are numerous pathways and molecules that are known to modulate VGCCs. These molecules include Ca^{2+} ions, hormones, neurotransmitters, enzymes, and various intracellular and extracellular proteins such as G-proteins, SNARE proteins, Ca^{2+}-binding and sensing proteins (Catterall, 2000). Some of these molecules differentially modulate various types of VGCCs, ultimately allowing only certain type of VGCCs to display specific physiological function, while having no effect on, or even preventing other types of VGCCs from exhibiting the same function. Among various VGCC modulators that have been identified, calmodulin serves as the prime example of VGCC regulator that differentially modulates VGCCs, including $Ca_v2.1$ and $Ca_v2.2$ channels.

Calmodulin (CaM) is a small Ca^{2+}-binding protein that is highly conserved and ubiquitously expressed in all Eukaryotic cells. CaM has two pairs of EF-hand motifs connected by a flexible linker, and it is a member of the superfamily of the EF-hand containing Ca^{2+}-sensing proteins. Each EF-hand domain, which is considered the most common Ca^{2+}-binding site, enables apoCaM to sense intracellular $[Ca^{2+}]$ and bind a total of four Ca^{2+} ions. This binding of Ca^{2+} ions is

required for the activation of CaM, which undergoes a conformational change in order to interact directly with other target proteins as shown in **Figure 2**. CaM is extensively studied for its remarkable Ca^{2+}-sensing power as well as the ability to regulate many important cellular processes by altering Ca^{2+} signals, modulating various ion channels, and acting as Ca^{2+} buffer. (Cheung, 1980; Stevens, 1983; Babu et al., 1985; Crivici and Ikura 1995; Yap et al., 1999; Lewit-Bentley and Rety, 2000; Haeseleer et al., 2002; Yáñez et al., 2012; Ben-Johny and Yue, 2013).

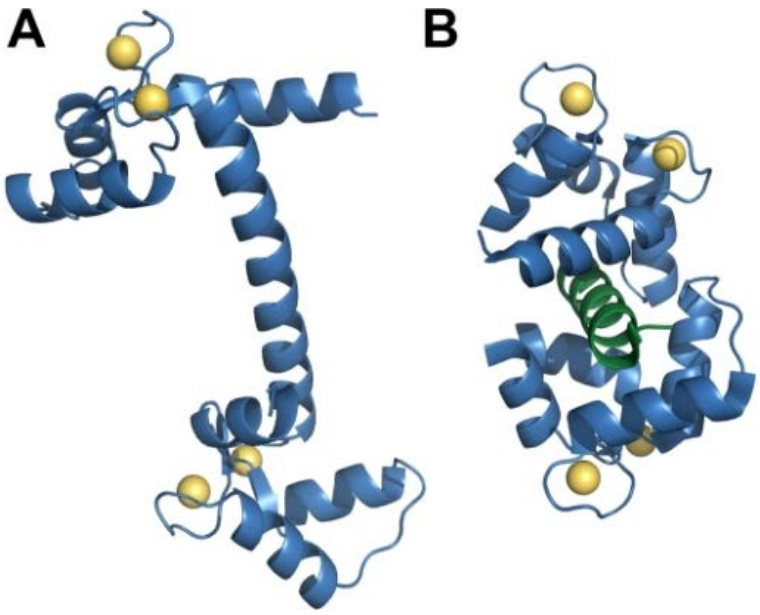


Figure 2. The Structure and Function of Ca^{2+}-Calmodulin Complex. (a) Calmodulin (Blue) has two homologous globular domains connected by a flexible linker. Each globular domain contains a pair of EF-hand motif that binds Ca^{2+} ions (yellow). (b) Binding of Ca^{2+} ions causes conformational change and activation of calmodulin, which directly interacts with its target protein (green) by binding at specific binding site. Adapted from Pepke et al., 2010.

Calmodulin modulates VGCCs by mediating two important feedback mechanisms called Ca^{2+}-dependent facilitation (CDF) and Ca^{2+}-dependent inactivation (CDI), both of which significantly contribute to the excitation-contraction coupling in the cardiac muscle and synaptic plasticity in the nervous system (Petegem et al., 2005; Zulke et al., 1999; Lee et al., 2003). During prolonged depolarizations in various synaptic terminals, the initial Ca^{2+} influx through VGCCs facilitates further Ca^{2+} entry in a positive feedback manner, progressively increasing the amplitude of Ca^{2+} currents. This Ca^{2+}-dependent facilitation is soon followed by the depression of the Ca^{2+} currents in a pulse-wise manner due to the inactivation of the channel as shown in **Figure 3 (**Cuttle et al., 1998; Lee et al., 1999; Catterall and Few, 2008). In presynaptic nerve terminals, it is crucial for VGCCs to transduce electrochemical signal to Ca^{2+} signals and allow neurotransmitter release in a rapid, efficient, and appropriate manner, and these feedback mechanisms allow fine-tuning of the Ca^{2+} signals for controlled and enhanced activation and inactivation of synaptic transmission (Weiss and Zamponi, 2012).

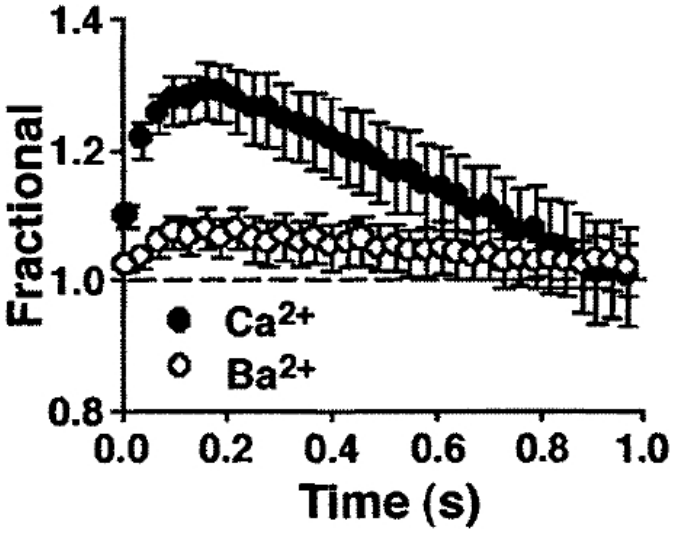


Figure 3. Ca^{2+}/CaM-Dependent Regulation of $Ca_V2.1$ VGCC. Only Ca^{2+} currents (Black dots) show initial Ca^{2+}-dependent facilitation followed by Ca^{2+}-dependent inactivation during repetitive depolarizations given at 100-Hz. The Ba^{2+} is not able to evoke either CDF or CDI. Adapted from Lee and Catterall, 2005.

CDF and CDI are mediated by calmodulin directly binding to specific, highly conserved cytoplasmic C-terminal domains (CTD) of the α_1 subunit of VGCCs, including a consensus IQ domain for interacting with CaM and a CaM-binding domain (CBD). When the IQ domain is mutated, VGCCs can no longer undergo CDF, since they are not able to bind CaM, and when the CBD is removed, both CDF and CDI are diminished in VGCCs (Lee et al., 1999, 2000; DeMaria 2001; Catterall and Few, 2008; Zühlke and Reuter, 1998; Qin et al., 1999).

It is important to note that these dual regulatory mechanisms are observed only in high-voltage activated (HVA) Ca_v1 and Ca_v2 channels, and not in low-voltage activated Ca_v3 channels. Also, only some types of these HVA channels exhibit both CDF and CDI, while some channels can only undergo CDI. For example, $Ca_v2.1$ channels display both CDF and CDI, while only CDI is observed in $Ca_v2.2$ channels. Despite overall conservation of the EF-hand-like (EF), pre-IQ-IQ, and CBD domains (**Figure 4**) which are crucial for allowing and regulating CDF of $Ca_v2.1$ channels, CDF is not observed in any splice variants of $Ca_v2.2$ channels as shown in **Figure 5** (Liang et al., 2003; Lee et al., 1999; DeMaria et al., 2001; Dunlap, 2007; Thomas et al., 2017).

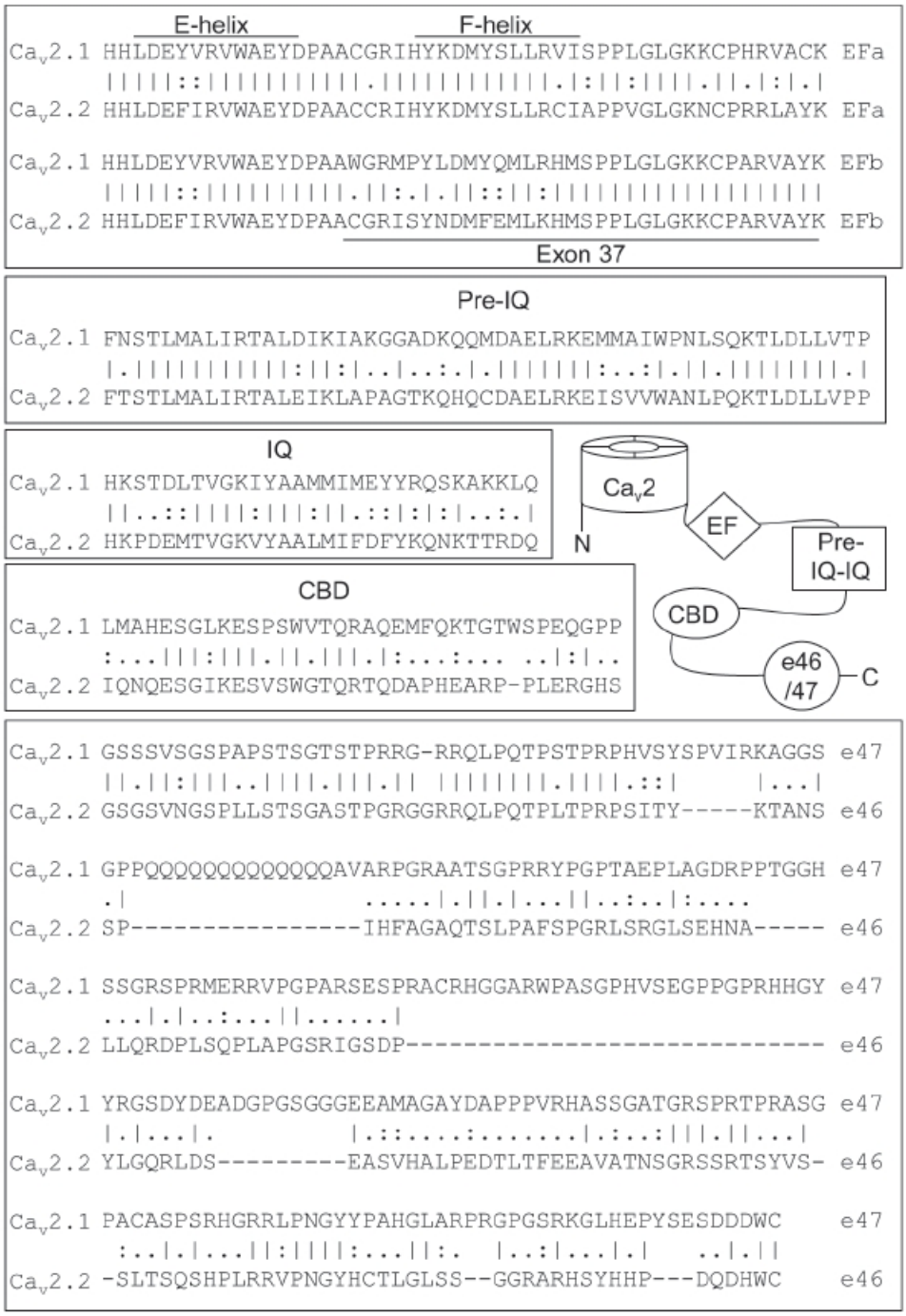


Figure 4. CDF Modulatory Domains in the CTD of $Ca_v2.1$ and Sequence Alignment with Analogous Regions of $Ca_v2.2$. Vertical bars (I), identical residues, colons (:) conservative substitutions; periods (.) nonconservative substitutions. Alignment is with human $Ca_v2.1$ and 2.2 sequences (Genbank # NM_023035.2, NM_001127222.1, NM_000718.3, and CM000671.2). Adapted from Thomas et al., 2017.

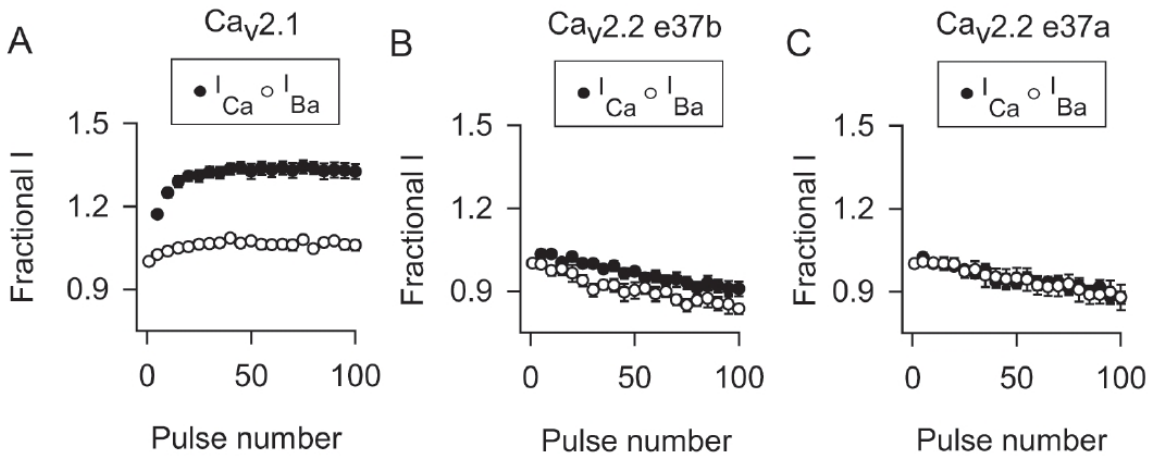


Figure 5. The Presence of CDF in $Ca_v2.1$ and the Absence of CDF in the Two Splice Variants of $Ca_v2.2$. The repetitive depolarizations cause CDF for $Ca_v2.1$ but not $Ca_v2.2$ channels. (A) CDF observed in $Ca_v2.1$ Channel. (B) No CDF is observed in $Ca_v2.2$ e37b splice variant. (C) No CDF is observed in $Ca_v2.2$ e37a splice variant. Adapted from Thomas et al., 2017.

Molecular Determinants Responsible for Ca^{2+}-Dependent Facilitation

The absence of CDF in $Ca_v2.2$ channel, as a result of differential regulation of VGCCs, may contribute to the differences in physiological roles of the two closely related $Ca_v2.1$ and $Ca_v2.2$ channels. However, the molecular determinants that prevent $Ca_v2.2$ channel from undergoing CDF have not been characterized only recently. Although the CDF-regulating CTD of $Ca_v2.1$ channel is conserved in the $Ca_v2.2$ channel, $Ca_v2.2$ channels do not undergo CDF as mentioned above, and this is due to differences in the amino acid sequences of the CTDs of $Ca_v2.1$ and $Ca_v2.2$ channels (Thomas et al., 2017).

Unlike the wild-type $Ca_v2.2$ channel, strong CDF is observed in the chimeric $Ca_v2.2$ channel containing the CTD of $Ca_v2.1$ channel ($Ca_v2.2$-$CT_{2.1}$), indicating that the amino acid sequence of the CTD of $Ca_v2.1$ channel is sufficient for allowing CDF. Also, in contrast to the wild-type $Ca_v2.1$ channel, CDF is abolished in the chimeric $Ca_v2.1$ channel containing the CTD of $Ca_v2.2$ channel ($Ca_v2.1$-$CT_{2.2}$) as shown in **Figure 6** (Thomas et al., 2017).

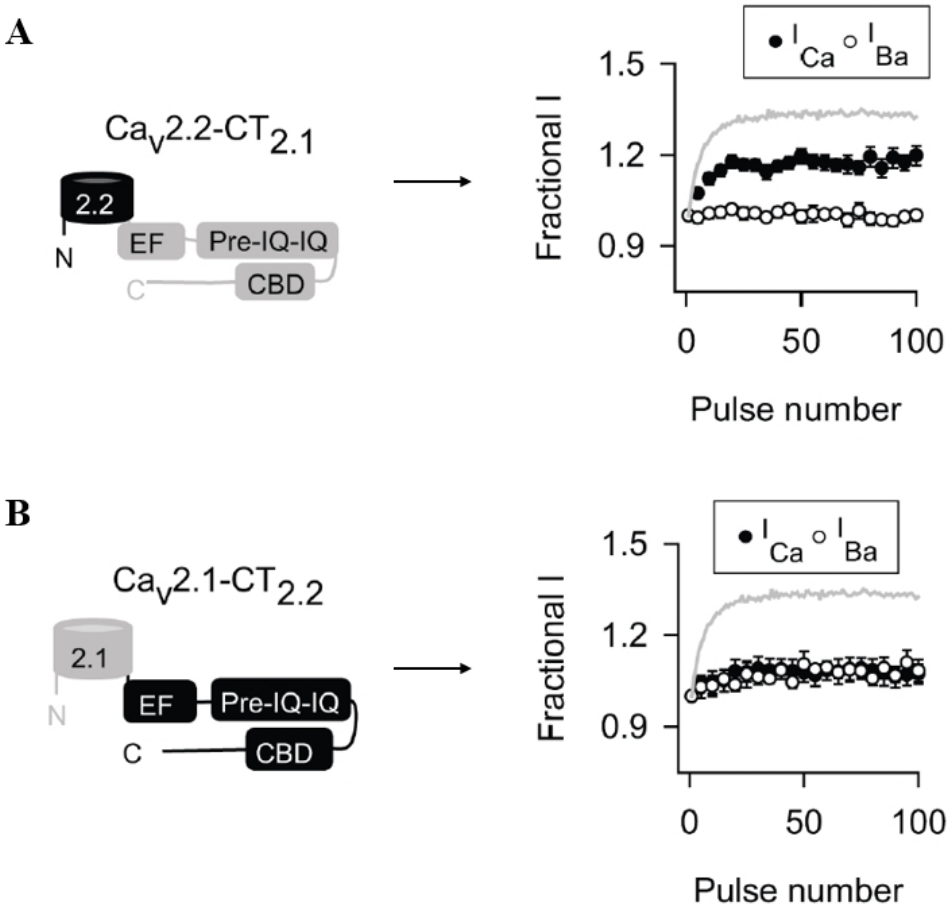


Figure 6. The CTDs of $Ca_v2.1$ and $Ca_v2.2$ Channels Determine Channel's Abilities to Undergo CDF. (A) The chimeric $Ca_v2.2$ channel containing the CTD of $Ca_v2.1$ channel displays robust CDF. (B) The chimeric $Ca_v2.1$ channel containing the CTD of $Ca_v2.2$ channel is not capable of undergoing CDF. Adapted from Thomas et al., 2017.

Additionally, only the proximal CTDs (EF through CBD) of $Ca_v2.1$ and $Ca_v2.2$ channels are known to determine the channel's ability to undergo CDF, while the distal CTDs of $Ca_v2.1$ and $Ca_v2.2$ has no effect on the channel's ability to undergo CDF. Just as seen previously in the chimeric $Ca_v2.2$ channel that contains the whole CTD of $Ca_v2.1$ channel ($Ca_v2.2$-$CT_{2.1}$), the chimeric $Ca_v2.2$ channel that contains only the proximal CTD of $Ca_v2.1$ channel ($Ca_v2.2$-$pCT_{2.1}$) display CDF (**Figure 7A**); the chimeric $Ca_v2.2$ channel that contains only the distal CTD of $Ca_v2.1$ channel ($Ca_v2.2$-$dCT_{2.1}$) is not able to undergo CDF (**Figure 7B**). Also, just as seen previously in the chimeric $Ca_v2.1$ channel that contains the whole CTD of $Ca_v2.2$ channel ($Ca_v2.1$-$CT_{2.2}$), CDF is abolished in the chimeric $Ca_v2.1$ channel that contains only the proximal CTD of $Ca_v2.2$ channel ($Ca_v2.1$-$pCT_{2.2}$, **Figure 7C**); CDF is not abolished in the chimeric $Ca_v2.1$ channel that contains only the distal CTD of $Ca_v2.2$ channel ($Ca_v2.1$-$dCT_{2.2}$) (**Figure 7D**). Thus, it is known that only the proximal CTD, but not distal CTD, of $Ca_v2.1$ channel allows the CDF, while the proximal CTD, but not distal CTD, of $Ca_v2.2$ channel disables the CDF (Thomas et al., 2017).

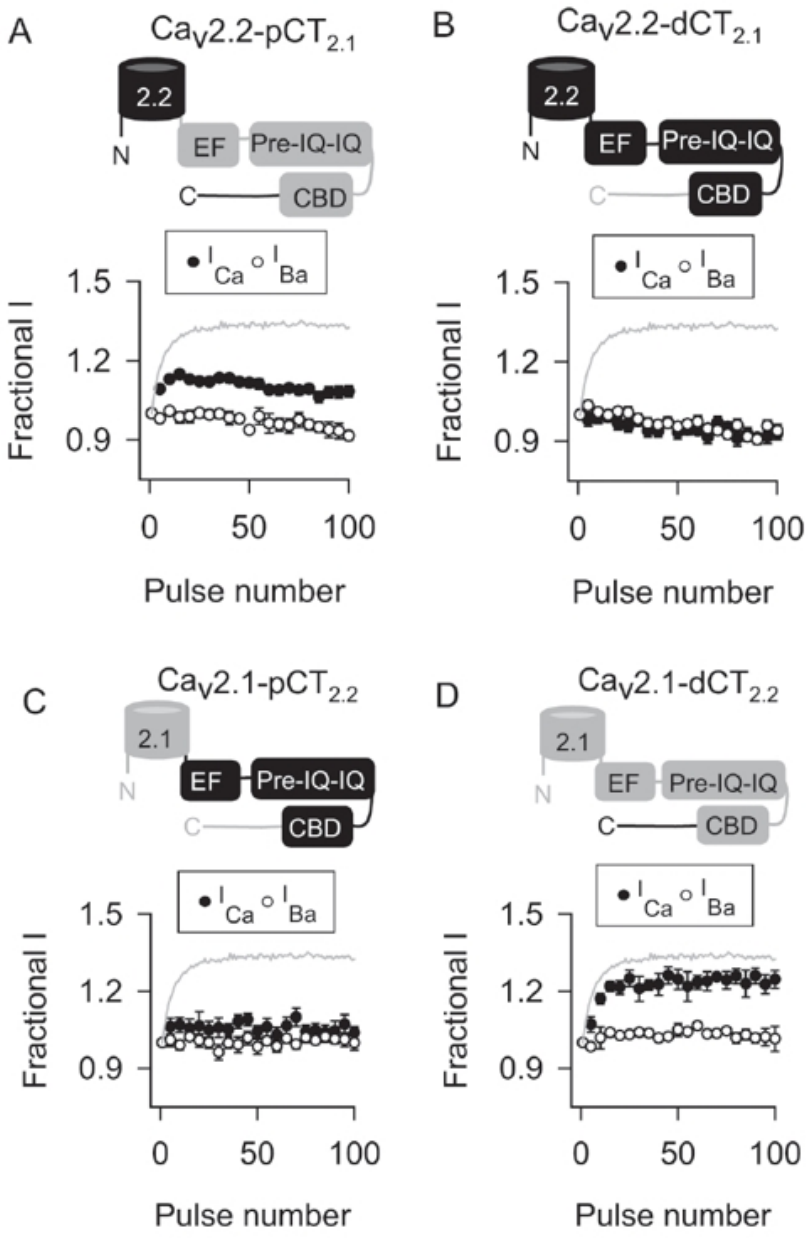


Figure 7. The proximal CTD of $Ca_v2.1$ Channel Contains CDF-Regulatory Domains which is not Functionally Conserved in the Proximal CTD of $Ca_v2.2$ channel. (A) CDF is observed in $Ca_v2.2$ channel containing proximal CTD of $Ca_v2.1$ channel. (B) CDF is absent in $Ca_v2.2$ channel containing distal CTD of $Ca_v2.1$ channel. (C) CDF is absent in $Ca_v2.1$ channel containing proximal CTD of $Ca_v2.2$ channel. (D) CDF is maintained in $Ca_v2.1$ channel containing distal CTD of $Ca_v2.1$ channel. Adapted from Thomas et al., 2017.

Lastly, within the proximal CTDs (EF-pre-IQ-IQ-CBD) of $Ca_v2.1$ and $Ca_v2.2$ channel, only EF and pre-IQ-IQ domains are known to affect the channel's ability to undergo CDF. When the EF, pre-IQ-IQ, and CBD domains of $Ca_v2.1$ channel are individually transferred to $Ca_v2.2$ channel, no CDF is observed (**Figure 8A, 8B,** and **8C**). Only when the EF and pre-IQ-IQ domains of $Ca_v2.1$ channel are transferred together, the chimeric $Ca_v2.2$ channel displays CDF (**Figure 9C**), indicating that the amino acid sequences encoding EF through pre-IQ-IQ domains within the proximal CTD of $Ca_v2.1$ channel are sufficient to evoke CDF in chimeric $Ca_v2.2$ channels. The dual transfer of either the pre-IQ-IQ and CBD domains together or the EF and CBD domains together does not evoke CDF in $Ca_v2.2$ channel (**Figure 9A** and **9B**). Conversely, the EF and pre-IQ-IQ domains of $Ca_v2.2$ channel, when transferred either individually or together, can eliminate CDF in $Ca_v2.1$ channel (**Figure 10A**, **10B,** and **10C**). The transfer of the CBD of $Ca_v2.2$ channel alone does not abolish CDF in $Ca_v2.1$ channel (**Figure 10D)**. All of the chimeric channels' ability to undergo CDF is summarized in **Table 3** (Thomas et al., 2017).

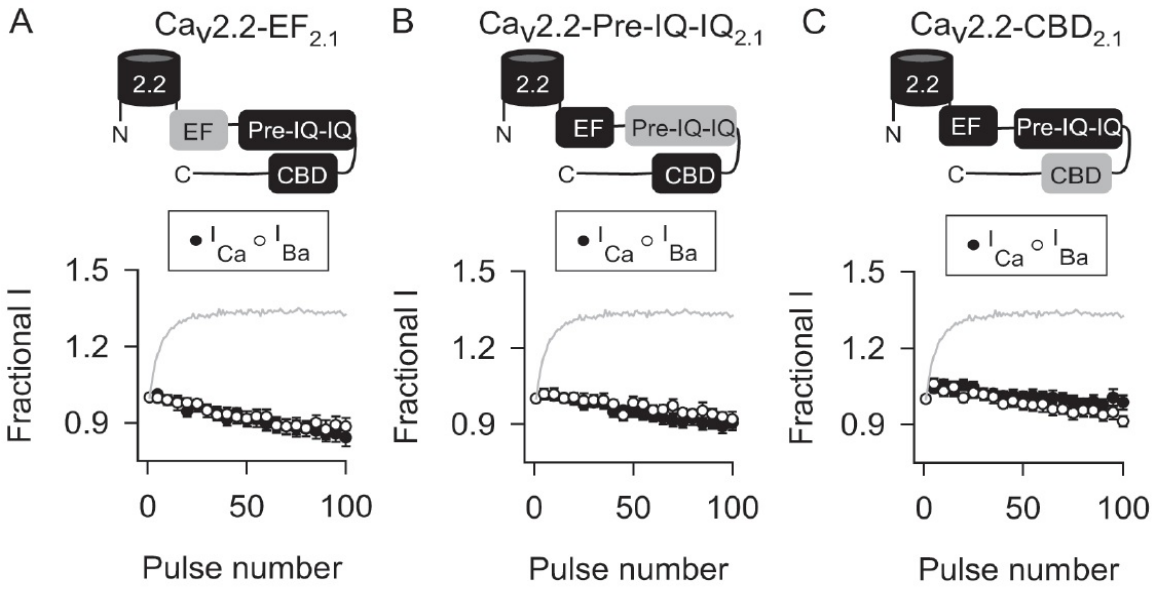


Figure 8. Each Individual Domain of $Ca_v2.1$ Channel is Not Sufficient to Allow CDF in $Ca_v2.2$ Channels. (A) $Ca_v2.2$ channel containing EF of $Ca_v2.1$ channel, (B) $Ca_v2.2$ channel containing pre-IQ-IQ of $Ca_v2.1$ channel, and (C) $Ca_v2.2$ channel containing CBD of $Ca_v2.1$ channel do not undergo CDF. Adapted from Thomas et al., 2017.

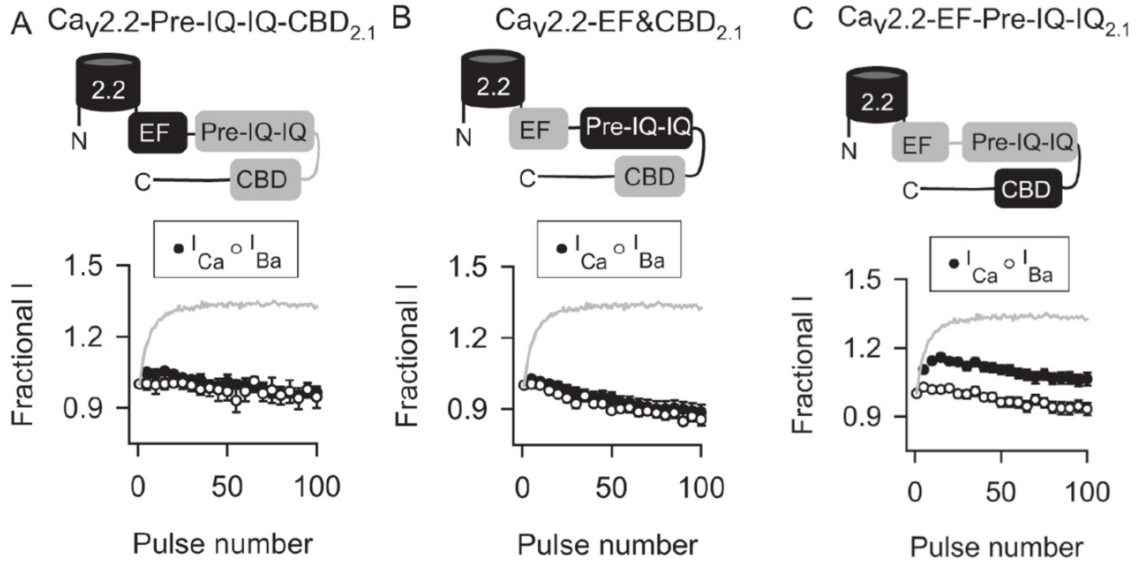


Figure 9. Both EF and Pre-IQ-IQ Domains of $Ca_v2.1$ Channel are Required to Evoke CDF in Cav2.2 channels. (A) $Ca_v2.2$ channel containing pre-IQ-IQ and CBD of $Ca_v2.1$ channel and (B) $Ca_v2.2$ channel containing EF and CBD of $Ca_v2.1$ channel does not undergo CDF. (C) Only $Ca_v2.2$ channel containing EF and pre-IQ-IQ of $Ca_v2.1$ channel undergoes CDF. Adapted from Thomas et al., 2017.

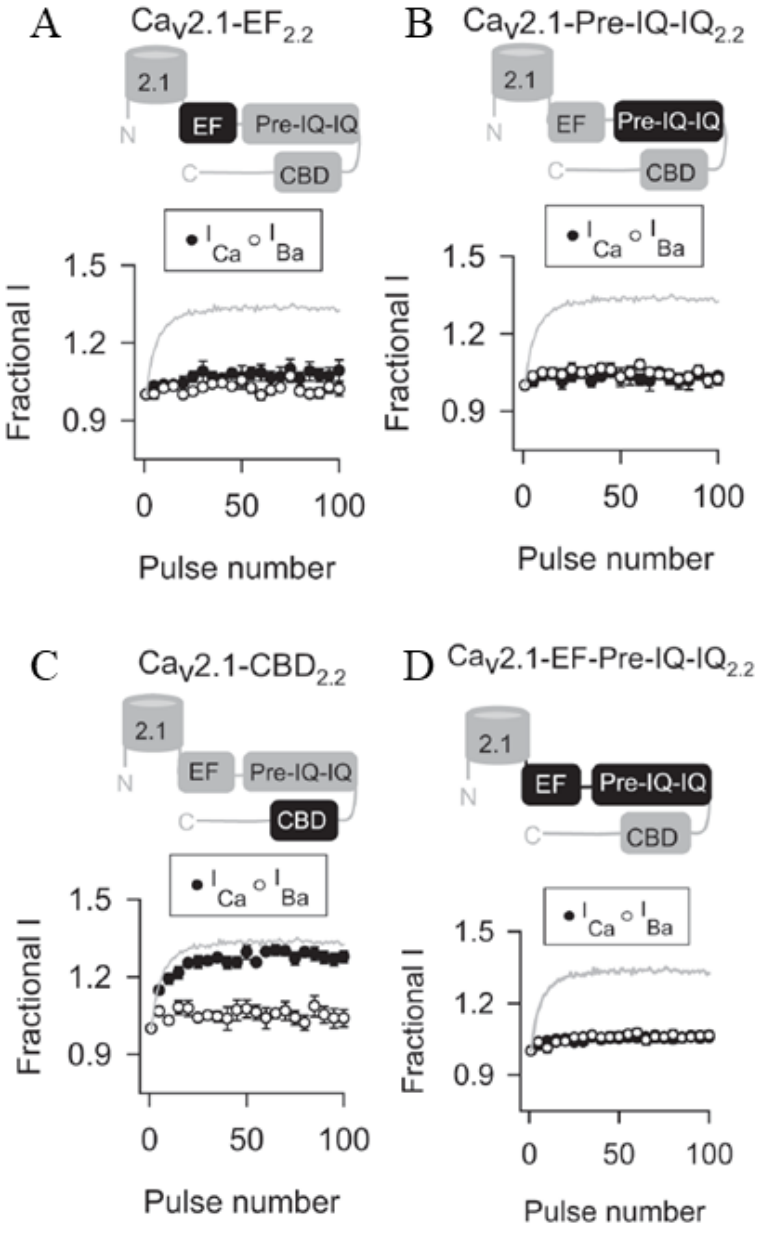


Figure 10. The EF and Pre-IQ-IQ Domains of $Ca_v2.2$ Channel Abolishes CDF in $Ca_v2.1$ Channel. (A) $Ca_v2.1$ channel containing EF domain of $Ca_v2.2$ channel does not undergo CDF. (B) $Ca_v2.1$ channel containing pre-IQ-IQ domain of $Ca_v2.2$ channel does not undergo CDF. (C) $Ca_v2.1$ channel containing CBD of $Ca_v2.2$ display CDF. (D) $Ca_v2.1$ channel containing both EF and pre-IQ-IQ domains of $Ca_v2.2$ dose not under CDF. Adapted from Thomas et al., 2017.

Table 3: The Summary of Chimeric Channels' Ability to Undergo CDF. (Thomas et al., 2017).

$Ca_v2.1$ Channel	**CDF?**	**$Ca_v2.2$ Channel**	**CDF?**
$Ca_v2.1$ WT	Yes	$Ca_v2.2$ WT	No
$Ca_v2.1$-$CT_{2.2}$	Disabled	$Ca_v2.2$-$CT_{2.1}$	Enabled
$Ca_v2.1$-$pCT_{2.2}$	Disabled	$Ca_v2.2$-$pCT_{2.1}$	Enabled
$Ca_v2.1$-$dCT_{2.2}$	Yes	$Ca_v2.2$-$dCT_{2.1}$	No
$Ca_v2.1$-$EF_{2.2}$	Disabled	$Ca_v2.2$-$EF_{2.1}$	No
$Ca_v2.1$-Pre-IQ-$IQ_{2.2}$	Disabled	$Ca_v2.2$-Pre-IQ-$IQ_{2.1}$	No
$Ca_v2.1$-$CBD_{2.2}$	Yes	$Ca_v2.2$-$CBD_{2.1}$	No
$Ca_v2.1$-EF&$CBD_{2.2}$	Disabled	$Ca_v2.2$-EF&$CBD_{2.1}$	No
		$Ca_v2.2$-Pre-IQ-IQ&$CBD_{2.1}$	No
		$Ca_v2.2$-EF&Pre-IQ-$IQ_{2.1}$	Enabled

The Role of EF-Hand Domain in Calmodulin Binding

As mentioned earlier, the pre-IQ-IQ domain is crucial for binding and transducing the effects of CaM on VGCCs (Pitt et al., 2001; Kim et al., 2004). It was previously shown that the association between the pre-IQ-IQ domain of $Ca_v2.2$ channel and CaM is weaker than the association between the pre-IQ-IQ domain of $Ca_v2.1$ channel and CaM (Peterson et al., 1999; Liang et al., 2003), suggesting that the lack of CaM-dependent CDF in $Ca_v2.2$ channel might be due to its pre-IQ-IQ domain's lower affinity for binding CaM. However, the effect of EF domain in CaM binding has not been studied—whether CaM binds differentially to the EF and pre-IQ-IQ domains with lower affinity in $Ca_v2.2$ channel compared to those of $Ca_v2.1$ channel.

Since EF domain is one of the two minimally required molecular determinants that support CDF in $Ca_v2.1$ channel and disable CDF in $Ca_v2.2$ channel, it is necessary to investigate the role of EF domain in CaM binding of VGCCs. This study examines the role of EF domain in regulating CaM binding to proximal C-terminal domains of $Ca_v2.1$ and $Ca_v2.2$ channels via pull-down binding assays.

SPECIFIC AIMS

The general objective of the study was to determine whether the absence of CaM/Ca^{2+}-dependent facilitation in $Ca_v2.2$ channel is due to the weaker binding of calmodulin. The specific goal was to investigate if calmodulin interacts with EF and pre-IQ-IQ domains of $Ca_v2.2$ channel with lower affinity compared to the same region of $Ca_v2.1$ channel. Also, the role of EF-hand domain in calmodulin binding to proximal C-terminal domains of $Ca_v2.1$ and $Ca_v2.2$ voltage-gated calcium channels is examined.

There are three specific aims:

1. Examine protein-protein interactions between calmodulin and pre-IQ-IQ domains of $Ca_v2.1$ and $Ca_v2.2$ channels using pull-down assays.
2. Examine protein-protein interactions between calmodulin and EF-pre-IQ-IQ domains of $Ca_v2.1$ and $Ca_v2.2$ channels using pull-down assays.
3. Compare calmodulin binding abilities of the EF-pre-IQ-IQ domains of $Ca_v2.1$ and $Ca_v2.2$ channels using pull-down assays.

METHODS

cDNAs and Molecular Biology

The following cDNAs were used: $Ca_v2.1$ (NM_001127221), $Ca_v2.2$ (AF055477), and rat calmodulin (rCaM1-148 [Pedigo and Shea, 1995], provided by M. Shea). For generating glutathione S-transferase (GST) fusion proteins, sequences corresponding to EF-pre-IQ-IQ and pre-IQ-IQ domains of $Ca_v2.1$ and $Ca_v2.2$ channels were amplified by polymerase chain reaction and cloned into BamHI and XhoI sites of the pGEX-4T-1 vector (Thomas et al., 2017).

Bacterial Transformation of DNAs

For bacterial transformations, fresh BL21 DE3 competent *Escherichia coli (E. coli)* bacteria was used. After tubes containing BL21 DE3 *E. Coli* cells were thawed on ice for 10 minutes, 1-5ul containing 100ng of each plasmid DNA (rCaM, $Ca_v2.1$EF-Pre-IQ-IQ, $Ca_v2.2$EF-Pre-IQ-IQ, $Ca_v2.1$Pre-IQ-IQ, and $Ca_v2.2$Pre-IQ-IQ) was added and mixed gently. The tubes containing bacteria and each plasmid DNA were kept on ice for 30 minutes, before they were placed on a pre-heated 42°C water bath for 10 seconds. After the heat shock, tubes were cooled down on ice for 5 minutes. Then, 500ul of room temperature SOC media was added to each tube. Tubes were incubated at 37°C and left vigorously rotating for 60 minutes. About 100ul of transformed bacteria were plated and

spread on warm selection plates, which were incubated overnight at 37°C for growth and selection.

Over-Expression of Proteins

A single colony of each transformed bacteria was picked to grow in 6ml of Luria Broth (LB) media with antibiotic overnight at 37°C with shaking (250 RPM) for each protein over-expression. In the following day, 5ml of each mini bacteria culture was added to 500ml of LB media with antibiotic and left shaken at 37°C until A_{600} readings reached between 0.4 and 0.6. When the A_{600} readings reached between 0.4 and 0.6, 500ul of 1M Isopropyl β-D-1thiogalactopyranoside (IPTG; final concentration of 1mM IPTG) was added for chemical induction of proteins. After 4 hours of induction at 30°C, cells were poured out into 1L bottles and centrifuged at 4,000 RPM for 20 minutes at 4°C. The pellets of cells were either used immediately for the protein purification or frozen on dry-ice and stored at -80°C until ready for purification.

Purification of GST-Tagged Proteins

The bacterial cell pellets were resuspended in ice-cold lysis buffer (50mM Tris-HCl, 150mM NaCl, pH 8.0) with protease inhibitors without EDTA and Phenylmethylsulfonyl fluoride (PMSF). After 20 minutes of rotation at 4°C, cells were lysed on ice with 4 rounds of sonication (15 seconds on, 30 seconds off at 35% amperage per round). After the sonication, the final concentration of 1%

Triton X-100 was added to sonicated samples, and cells were incubated at 4°C again, rotating for 30 minutes. After the incubation, samples were centrifuged at 14,000 RPM, 4°C for 10 minutes to isolate and remove insoluble materials. Soluble supernatants were transferred to pre-chilled tubes containing pre-washed, alcohol-free GST beads (GE Healthcare Life Sciences), and incubated at 4°C, rotating overnight. On the following day, samples were centrifuged at 700 x G for 2 minutes, and supernatants were carefully removed. GST beads were washed 3 times with lysis buffer to remove as much unwanted and unbound proteins as possible. The expressions of the GST-tagged proteins were confirmed by running the purified proteins on 4-12% SDS-Page gel with MES buffer. Purified protein samples were stored at 4°C until ready for binding assay, and the concentrations of GST-tagged proteins were measured.

Purification of Calmodulin (Adapted from Theoharis et al., 2008)

The bacterial cell pellet with over-expressed CaM was resuspended in ice-cold lysis buffer (20mM Tris-HCl, 1mM EDTA, pH7.2). Resuspended cells were lysed on ice using 2 rounds of sonication (5 second on, 10 seconds off for 60 seconds at 35% amperage per round). After the sonication, cells were incubated at 4°C for 15 minutes, and after the incubation, cells were centrifuged at 14,000 RPM and 4°C for 30 minutes to isolate and remove insoluble materials. The soluble supernatant was transferred to 50mL conical tubes and 1M $CaCl_2$ was added to a final concentration of 10mM. The sample was then treated with heat

using a pre-heated water bath for 10 minutes at 80°C. After the sample was taken out, it was allowed to cool slowly at room temperature. The cooled sample was centrifuged at 4,000 RPM and 4°C for 15 minutes. The supernatant, which contains heat-treated CaM, was filtered using 0.22 µm syringe filter unit and was placed on ice. Meanwhile, the phenyl-sepharose column was prepared for column chromatography purification of calmodulin. All of the following steps were performed in sterile 4°C cold room to prevent any possible bacterial contamination and degradation of protein. The phenyl-sepharose column was washed with 1 column volume of filter-sterilized distilled water to remove any trace of ethanol. The column was then equilibrated with 3 column volumes of Buffer A (50mM HEPES, 10mM $CaCl_2$, 3mM DTT, pH 7.4, filter-sterilized with 22 µm filters), before the supernatant containing CaM was loaded to the column to be bound by the phenyl-sepharose beads by gravity flow. The column was washed with 3 column volumes of Buffer A, 3 column volumes of Buffer B (50mM HEPES, 0.5M NaCl, 10mM $CaCl_2$, 3mM DTT, pH 7.4, filter-sterilized with 22 µm filters), and 3 column volumes of Buffer A again. Finally, CaM was eluted two times with 1 column volume of Buffer C (50mM HEPES, 1mM EDTA, 3mM DTT, pH 7.4, filter-sterilized with 22 µm filters). Some aliquots of eluted CaM were stored -20°C and some aliquots of CaM were stored at 4°C until ready for binding assay. CaM sample that was stored at 4°C for more than a month was discarded. The concentration of CaM was measured by using both the bicinchoninic acid

assay and spectrophotometer. The expression of CaM was confirmed by SDS-PAGE gel electrophoresis and Coomassie staining as shown below (**Figure 11**).

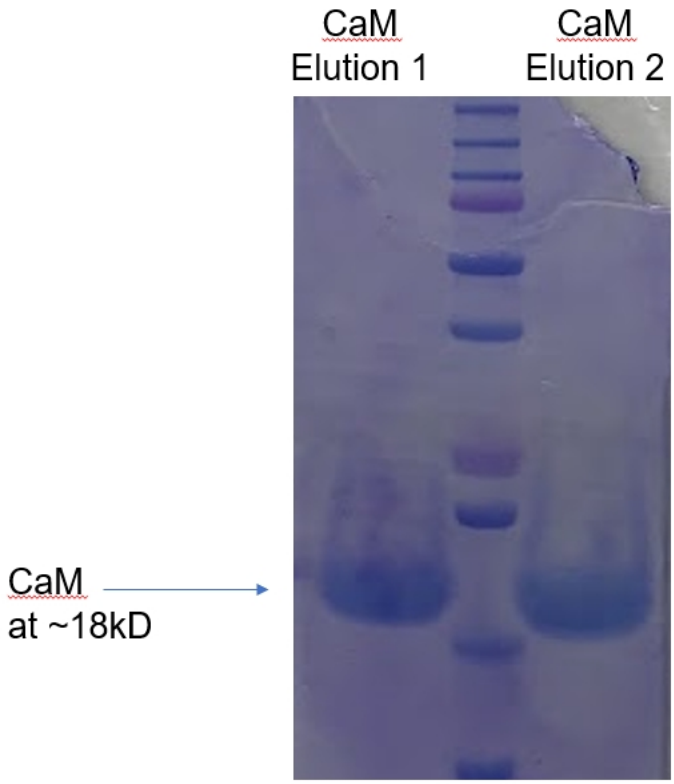


Figure 11. Expression of Calmodulin. Calmodulin (18kD) is over-expressed and purified using phenyl-Sepharose column chromatography.

Pull-Down Binding Assays

Purified CaM (1-10ug) was added to GST or GST-tagged $Ca_v2.1$ or $Ca_v2.2$ proteins (EF-pre-IQ-IQ and pre-IQ-IQ; 5ug) immobilized on glutathione Sepharose beads. The reaction was brought to a total volume of 750 µL in 1.5mL Eppendorf tube with binding buffer (20mM Tris-HCl, pH 7.3, 2mM $CaCl_2$, ± 150mM NaCl; results were similar with or without the added NaCl and so were combined). Binding reactions were incubated at 4°C, rotating for 1 hr. The beads

were washed three times with 1 mL of ice-cold binding buffer, and bound proteins were eluted in elution buffer (DTT reducing agent, lithium dodecyl sulfate, pH 8.4 [Thermo Fisher Scientific] in dH_2O).

Immunoblotting

Eluted protein samples were heated at ~95°C for 10 minutes and loaded into NuPAGE 4-12% Bis-Tris polyacrylamide gels (Thermo Fisher Scientific). MES buffer was used to run gels at 120 volts for 2-2.5 hours. After the samples were resolved by SDS-PAGE, proteins were transferred to nitrocellulose membrane at 100 volts for 1.5 hour. After transfer was complete, the nitrocellulose membrane was rinsed with dH_2O and stained with Ponceau S to detect the GST-proteins. Ponceau stained blot was scanned for quantitative densitometry analysis, before it was rinsed with dH_2O again. The blot was blocked in 5 mL of 4% milk in TBST (0.1% Tween-20 in TBS) for 1 hour at room temperature, and then incubated with primary rabbit polyclonal antibodies against CaM (1:1,000, 301 003, RPID:AB_2620046; Synaptic Systems) for 1 hour at room temperature. After the primary antibody incubation, the blot was rinsed 4 times in TBST for 5 minutes per rinse. After being rinsed, the immunoblot was incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG, 1:4,000, I5006, RRID: AB_1163659; Sigma-Aldrich) for 1 hour at room temperature. The blot was rinsed four times again with TBST and was

processed with reagents for enhanced chemiluminescent detection (Thermo Fisher Scientific) before being exposed to an autoradiography film (Denville).

Quantitative Analysis

For quantitative analysis, densitometry was performed using a Canon LIDE 200 scanner and ImageJ (NIH) software. The Western blot signal for CaM was normalized to the signal corresponding to the Ponceau-stained GST fusion proteins. Results from at least three independent experiments were pooled for statistical analysis (ANOVA and post hoc Turkey tests).

RESULTS

Stronger interaction with CaM was expected in the pre-IQ-IQ region of $Ca_v2.1$ channel than of $Ca_v2.2$ channel as described previously (Liang et al., 2003). As expected, the pull-down assay results showed significantly stronger interaction of calmodulin with the pre-IQ-IQ region of $Ca_v2.1$ channel compared to the same region of $Ca_v2.2$ channel (**Figure 12A, B**). CaM did not interact with the GST control. Surprisingly, when the EF-hand domain was added to the pre-IQ-IQ domain of $Ca_v2.2$ channel, CaM binding was significantly enhanced, and there was no statistically significant difference in CaM binding to the EF-pre-IQ-IQ region of $Ca_v2.2$ channel and of $Ca_v2.1$ channel (**Figure 12A, B)**. The addition of the EF-hand domain to the pre-IQ-IQ domain of $Ca_v2.1$ channel did not affect CaM binding, as there was no significant difference in CaM binding of the pre-IQ-IQ domain and the EF-pre-IQ-IQ domain of $Ca_v2.1$ channel—CaM interacted just as well in both the presence and absence of EF-hand in $Ca_v2.1$ channel (**Figure 12A, B)**.

To observe the effect of the EF-hand domain in interaction of $Ca_v2.2$ channel with CaM more clearly, increasing concentrations of CaM were used in pull-down binding assays (1, 2.5, 5, and 10 µg). For all concentrations of CaM tested, the amount of CaM bound to the $Ca_v2.2$ Pre-IQ-IQ was only ~20% of that to the $Ca_v2.1$ Pre-IQ-IQ (**Figure 13A, B**), and there was no statistically significant difference in interaction between CaM and the EF-Pre-IQ-IQ of $Ca_v2.1$ and $Ca_v2.2$ channels (**Figure 13C, D**).

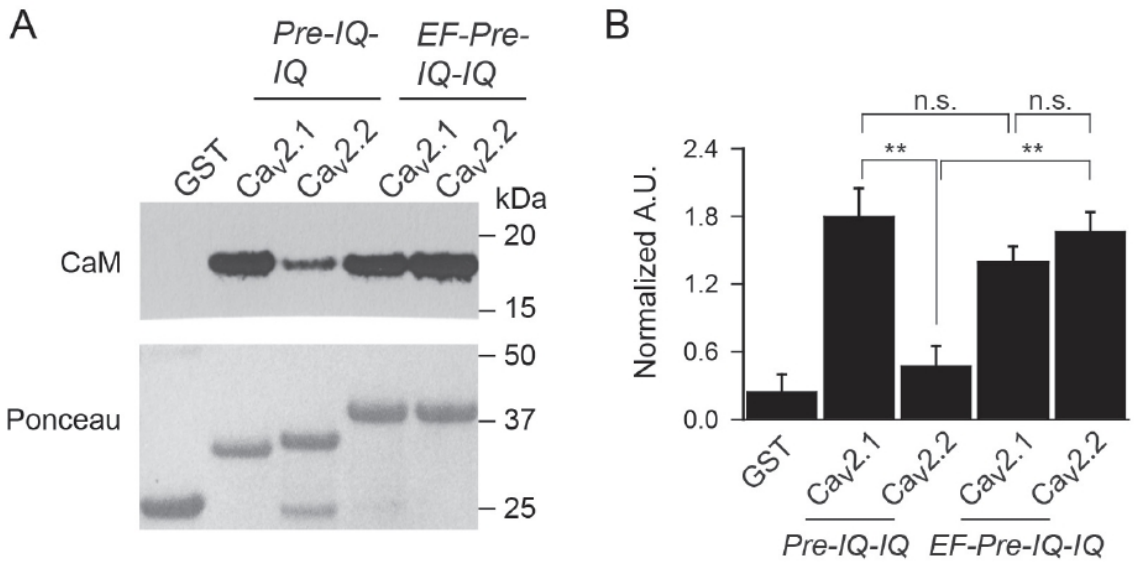


Figure 12. CaM Differentially Binds to Pre-IQ-IQ and EF-pre-IQ-IQ of $Ca_v2.1$ and $Ca_v2.2$. (A) GST and GST-tagged Ca_v2 proteins (5µg) were incubated with CaM (2.5µg), and bound CaM was detected by Western blotting. Ponceau staining indicated the amount of each GST protein in the reactions. (B) The signal intensity corresponding to CaM was normalized to that for the GST-protein. A.U., arbitrary units; n.s., not significant; **, $P < 0.001$, n=4, one-way ANOVA and post-hoc Turkey test. Data are representative of four independent experiments. (Data published in *the Journal of General Physiology*; Thomas et al., 2017).

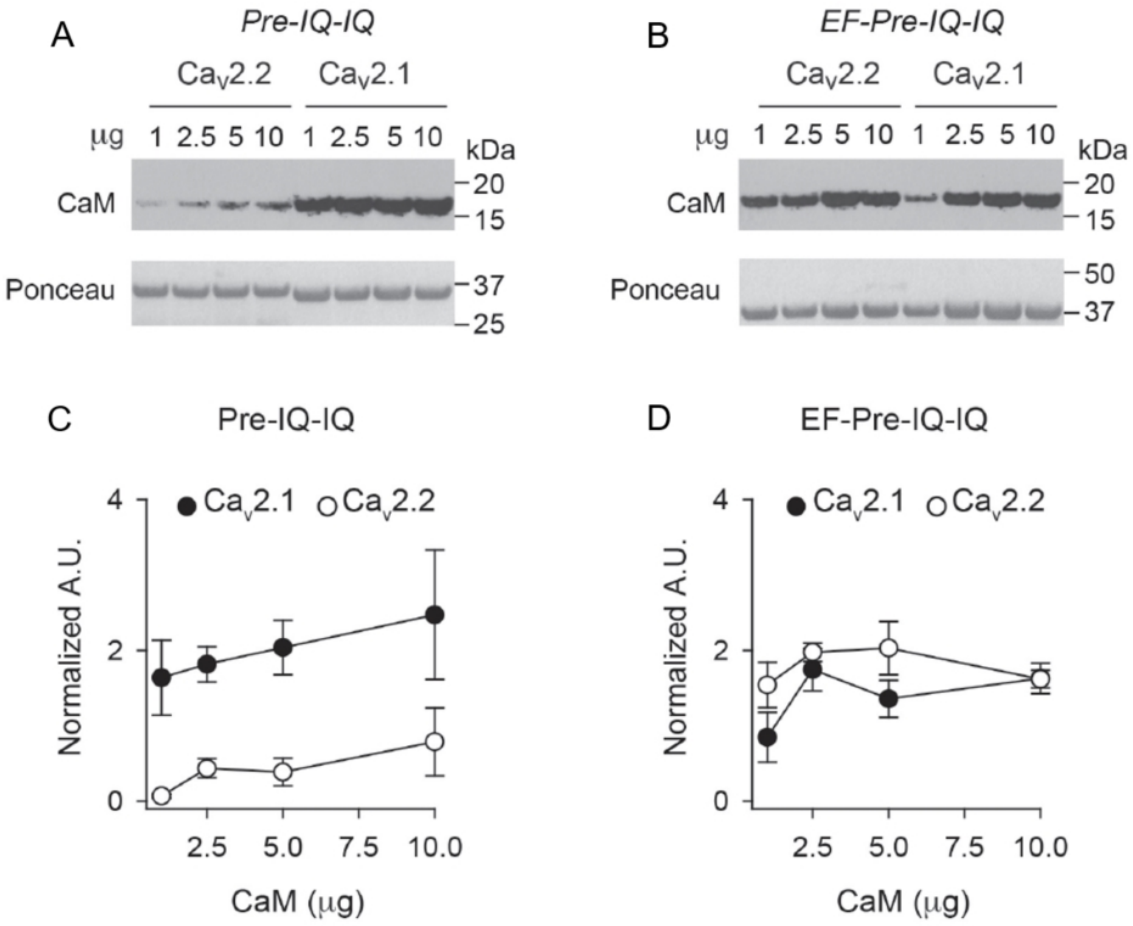


Figure 13. The Effect of EF-hand in Differential Binding of CaM. As in **Figure 12 (A) and** (**B)** except that variable amounts of CaM (1-10µg) were used in the assay. In (C) and (D), data were analyzed by two-way ANOVA. There was a significant difference in results obtained for pre-IQ-IQ ($P < 0.01$; n=3) but not EF-pre-IQ-IQ ($P = 0.75$; n=3) of $Ca_v2.1$ and $Ca_v2.2$. Data are representative of three independent experiments. Data represent mean ± SEM.
(Data published in the *Journal of General Physiology;* Thomas et al., 2017)

DISCUSSION

Through the pull-down binding assays, the EF-hand domain was found to enhance interaction between CaM and the proximal C-terminal domain of $Ca_v2.2$ channel. When the EF-hand domain was added, the weak interaction between CaM and the pre-IQ-IQ domain of $Ca_v2.2$ channel (**Figure 12**, DeMaria et al., 2001; Liang et al., 2003) became as strong as the interaction between CaM and the proximal C-terminal domain of $Ca_v2.1$ channel, suggesting that the EF-hand domain plays a distinct role in differentially regulating CaM binding of $Ca_v2.1$ and $Ca_v2.2$ channels. The equal CaM binding strengths of the EF-pre-IQ-IQ domains of $Ca_v2.2$ and $Ca_v2.1$ channels (**Figure 12**) suggest that the absence of CDF in $Ca_v2.2$ channel does not arise simply from decreased affinity for CaM binding, as hypothesized previously (Liang et a., 2003). Rather, the lack of CDF in $Ca_v2.2$ channel may arise due to its EF-pre-IQ-IQ domain's inability to fully transduce the effects of CaM. The EF-hand domain of $Ca_v2.2$ channel might interact with CaM bound to the pre-IQ-IQ domain in ways that prevent CaM from undergoing a conformational change that allows CDF. The EF-hand might also interact with other regions of the channel, namely, linker domains of cytoplasmic loops I and II (Kim et al., 2004) and III and IV (Wu et al., 2016) in ways that prevent a conformational change of the channel that is favorable for CDF. Future studies may shed light on a knowledge of how the EF-hand domain interacts together with other parts of the channel to transduce the effects of CaM and regulate CDF.

As described previously, Ca_v1 and Ca_v2 channels are differentially modulated by CaM (Liang et al., 2003), despite overall conservation of amino acid sequences encoding EF-hand through pre-IQ-IQ domains. This is evidenced by the ubiquitous presence of CDI and selective presence of CDF in HVA VGCCs (Budde et al., 2002; Liang et a., 2003). The reason why CDF is not supported in $Ca_v2.2$ channel remains uncertain, but it may involve the EF-hand domain acting as one of the safeguards that prevent unnecessary and excessive amplification of pain signals during nociception caused by a rapid increase in cytoplasmic [Ca^{2+}] as well as prolonged elevation of intracellular [Ca^{2+}] in the peripheral nervous system (Hirning et al., 1988). Also, since $Ca_v2.2$ channel plays a secondary role to $Ca_v2.1$ channel at many synapses of central nervous system, the absence of CDF in $Ca_v2.2$ channel may allow itself to work in a less aggressive way, or even counteract the effect of $Ca_v2.1$ channel. In this paper, we examined the significance of EF-hand domain in calmodulin binding of $Ca_v2.1$ and $Ca_v2.2$ voltage-gated calcium channels.

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